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Improved Flotation Technique for Microscopy of 'In situ' Soil and Sediment Microorganisms

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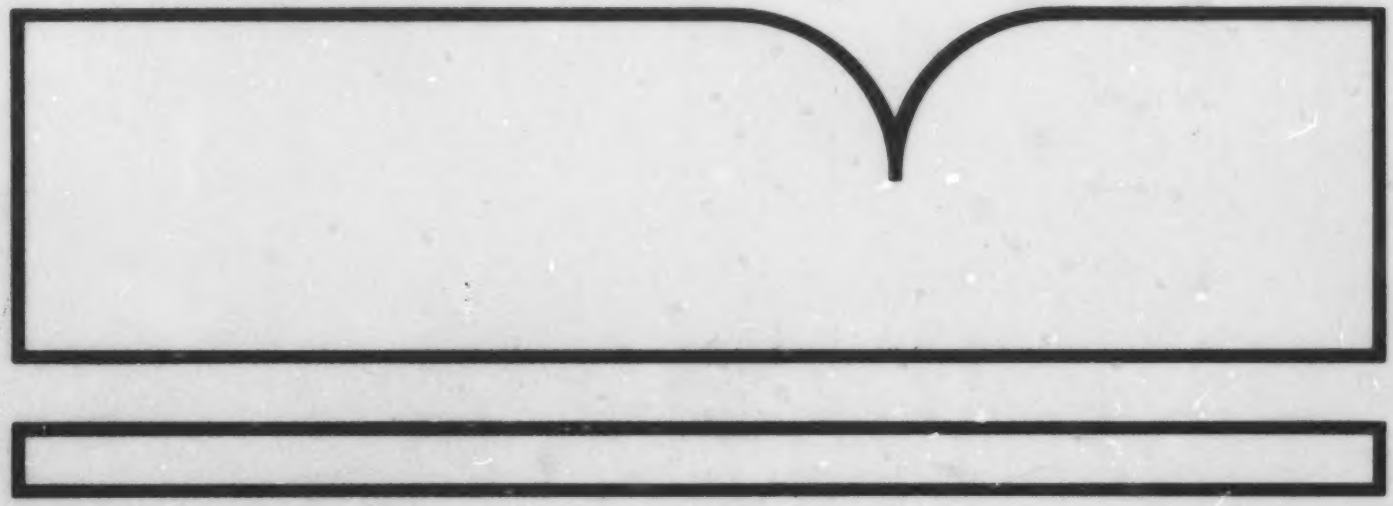

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16. ABSTRACT

An improved flotation method for microscopical examination of in situ soil and sediment microorganisms was developed. Microbial cells were released into gel-like flotation films that were stripped from soil and sediment aggregates as these aggregates were submerged in 0.5% solutions of poly-vinylpryrrolidone (PVP). The use of PVP solutions instead of water facilitated the release of films from saturated samples like aquifer sediments, as well as from typical surface soils. In situ microbial morphological characteristics could then be surveyed rapidly by light microscopical examination of films stained with acridine orange. This method effectively determined the range of morphological diversity in a variety of sample types. It also served to detect microcolonies and other spatial relationships between microbial cells. Only a small fraction (3.4-10.1%) of the microflora was released into the flotation films, but plating and direct microscopical evaluations showed that this fraction was representative of the total population.

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Improved Flotation Technique for Microscopy of In Situ Soil and Sediment Microorganisms

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An improved flotation method for microscopy of in situ soil and sediment microorganisms was developed. Microbial cells were released into gellike flotation films that were stripped from soil and sediment aggregates as these aggregates were submerged in 0.5% solutions of polyvinylpyrrolidone. The use of polyvinylpyrrolidone solutions instead of water facilitated the release of films from saturated samples such as aquifer sediments as well as from typical surface soils. In situ microbial morphological characteristics could then be surveyed rapidly by light microscopy of films stained with acridine orange. This method effectively determined the ranges of morphological diversity in a variety of sample types. It also detected microcolonies and other spatial relationships among microbial cells. Only a small fraction (3.4 to 10.1%) of the microflora was released into the flotation films, but plating and direct evaluations by microscopy showed that this fraction was representative of the total population.

In 1973, Waid (11) described a method for direct examination of microbial cells from soil by transmission electron microscopy (TEM). This method was an adaptation of the flotation technique used by Grossman and Lynn (8) to size soil particles. Small soil particles and microbial cells were present in gellike films that were stripped off the surfaces of soil aggregates as they were submerged in water. Waid retrieved the films on coated specimen grids and prepared them for TEM by negative staining or metal shadowing (11, 12). This simple and rapid procedure allows observation of in situ soil microorganisms without growing them in the laboratory.

Efforts to study microorganisms in natural habitats by direct observation were recently applied to the subsurface sediments (aquifers) in which groundwater is found (2, 3, 6, 7, 13). The organisms in aquifer sediments can be observed with release and concentration methods originally designed to prepare microorganisms in surface soils for thin sectioning and TEM (1, 4, 5, 9). The Waid technique has been comparatively ineffectual with aquifer samples because the water used for flotation seldom strips coherent films from saturated soil materials and cell numbers frequently are so low that observation of unconcentrated specimens by TEM is very time-consuming. Yet, the need for a simple Waid-type procedure for use on aquifer materials is great because the release and concentration methods now used to observe aquifer bacteria are exceedingly tedious.

The purpose of this study was to modify the Waid technique to improve its effectiveness on aquifer and other saturated sediment samples. This goal was achieved by substituting a polyvinylpyrrolidone (PVP) solution for distilled water as the flotation fluid. In addition, light microscopy of films stained with acridine orange (AO) facilitated observation of films with low cell numbers. These changes resulted in a technique that can be applied to a wide variety of saturated and unsaturated sediments and soils.

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MATERIALS AND METHODS

Soil and sediment samples. Surface soils and subsurface sediments from four sites were used to evaluate the flotation procedure described below. This set of samples (Table 1) included unsaturated and saturated (aquifer) materials that contained a wide range of microbial cell numbers. The subsurface sediments were obtained aseptically (13) by scientists at the U.S. Environmental Protection Agency Robert S. Kerr Environmental Research Laboratory, Ada, Okla.

Viable cell counts. Viable cell numbers in all samples were estimated by plate counting. A 10-g sample was blended with 100 ml of sterile 0.1% sodium PP_i ($Na_4P_2O_7 \cdot 10H_2O$) for 1 min in a Waring blender to produce a well-dispersed suspension. Serial dilutions of this suspension (in 0.1% $Na_4P_2O_7 \cdot 10H_2O$) were then spread plated in triplicate on dilute PTYG medium which contained the following ingredients per liter of distilled water: peptone, 0.25 g; tryptone, 0.25 g; yeast extract, 0.5 g; glucose, 0.5 g; $MgSO_4 \cdot 7H_2O$, 30 mg; $CaCl_2 \cdot 2H_2O$, 3.5 mg; Bacto-Agar (Difco Laboratories), 15.0 g. The plates were incubated aerobically for 1 to 8 weeks at 18 to 22°C and then evaluated; the total number of colonies was counted, and in some cases, each of the recognizably distinct types of colonies present at the statistically countable dilution was characterized.

Flotation procedure. Flotation films were produced with a technique similar to that described by Grossman and Lynn (8). Büchner funnels (no. 2 and 3) were used that were connected by 46 cm of gum rubber tubing attached to their stems (Fig. 1). The funnels rested in burette clamps positioned approximately 18 and 12 cm above the base of a ring stand. The sample was placed in the no. 2 funnel, and 50 ml of fluid (see below) was poured into the no. 3 funnel. The no. 3 funnel was then slowly raised to a position above the no. 2 funnel (30 cm above the base of the stand); as this was done, the fluid flowed up through the tubing into the no. 2 funnel and over the sample, thereby causing the films to form on the fluid surface directly above the sample. All parts of the

TABLE 1. Soil and subsurface sediment samples

Sample no.	Sampling site	Collection date	Sample type[a]	Saturation status[b]	Depth (m)	Viable cells (CFU [SD] g [wet wt]$^{-1}$)[c]
E9B/1	Albany, Ga.	06/10/83	SF	U	0.25	$2.7\ (1.1) \times 10^6$
E9B/2	Albany, Ga.	06/10/83	SF	I	0.65	$5.7\ (0.7) \times 10^5$
E9B/3	Albany, Ga.	06/10/83	SS	S	1.20	$7.9\ (0.6) \times 10^3$
9U	Lula, Okla.	01/18/85	SF	U	—[d]	$6.0\ (1.8) \times 10^7$
9S4	Lula, Okla.	01/16/85	SF	U	0.22	$9.8\ (1.9) \times 10^5$
9S1	Lula, Okla.	01/16/85	SS	U	1.32	$9.9\ (0.9) \times 10^5$
9S8	Lula, Okla.	01/16/85	SS	S	7.57	$8.2\ (2.3) \times 10^7$
5G1B	Conroe, Tex.	06/16/83	SS	S	7.36	$<10^3$
14J13	Conroe, Tex.	06/13/83	SS	S	7.80	$<10^3$
16C4	Conroe, Tex.	06/16/83	SS	S	7.52	$<10^2$
16D1	Conroe, Tex.	06/16/83	SS	S	5.87	$<10^3$
K1	Tallahassee, Fla.	07/13/84	SF	U	—	$5.2\ (0.7) \times 10^6$
T1	Tallahassee, Fla.	07/13/84	SF	U	—	$1.5\ (0.3) \times 10^8$

[a] SF, Surface soil (A horizon material); SS, subsurface sediment below the A horizon.
[b] U, Unsaturated material above the local water table; S, saturated zone below the water table; I, interface zone at the upper surface of the water table.
[c] Average of triplicate spread plates, containing dilute PTYG medium.
[d] —, Surface soil.

apparatus that contacted samples or fluid were precleaned and autoclaved.

The primary modification of the Waid approach (11, 12) devised in this study was the substitution of a PVP solution for distilled water as the flotation fluid. Several other flotation fluids (1% $Na_4P_2O_7 \cdot 10H_2O$, 0.1 N HCl, 0.1 N NaOH, 0.1% Trition X-100, 1% disodium EDTA, 20% glycerol, 1 M sucrose, and 30% $ZnSO_4$) were tested initially, but none of them produced noticeably better results than did water; consequently, PVP solutions were used in all subsequent investigations. The film-release efficacy of PVP solutions was compared with that of water in producing films from several different soil and sediment samples (see Results). The amount of film produced by each fluid was judged visually. Similar experiments were done that compared the three different-molecular-weight PVPs available (PVP-10, -40, and -360 with molecular weights of 10,000, 40,000, and 360,000, respectively) and determined the effects of PVP concentration on film release. A 0.5% solution of PVP-360 was used for all subsequent studies.

Microscopy of films. Some films were prepared for TEM so that they could be compared to the water-produced films described by Waid (11). These films were picked up with Parlodion-coated TEM grids (copper, 200 mesh), dried, shadow coated with platinum-carbon, and then examined with a Philips EM-201 transmission electron microscope.

Films were also stained with AO and examined by epifluorescence light microscopy instead of TEM. Films were picked up for light microscopy by carefully lowering a sterile cover slip parallel to the film until the film adhered to the glass surface. Excess fluid was drained off, and the film was allowed to air dry under conditions that precluded airborne contamination (under a sterile, inverted petri dish). The dried film was stained for 2 min with several drops of filter-sterilized 0.01% AO (aqueous) and washed with distilled water. The stained cover slip was placed sample side down on a drop of sterile water on a clean, sterile slide. Excess water was drawn out from under the cover slip with bibulous paper, and the edges of the cover slip were sealed with wax to prevent further drying.

Data on microbial morphology and morphological diversity were obtained by examination of AO-stained films by epifluorescence light microscopy in a Nikon Optiphot microscope fitted with a 100-W high-pressure Hg source, a Nikon B excitation filter/dichroic mirror, and an HFX automatic camera system. Representative fields were photographed with Kodak Ektachrome 400 or Kodak Technical Pan film. The micrographs were then analyzed for cell sizes, shapes, and numbers, abundance of different morphological types, presence of microcolonies, and general spatial relationships among cells with different morphologies.

AODCs. Selected samples were examined by an AO direct count (AODC) procedure (10), as modified by Ghiorse and Balkwill (6). Morphological observations made during counting were then compared to those made by light microscopy of AO-stained flotation films.

Effect of PVP on microbial growth. The potential toxicity of PVP to microorganisms was tested by determining its effects on the growth of two subsurface bacterial strains, pH2-B and 82-D. Strain pH2-B is a small, gram-negative coccobacillus that was isolated from an aquifer at Conroe, Tex.; strain 82-D is a gram-positive rod/coccus that was isolated from an aquifer at Lula, Okla. (3). Each of these strains was inoculated into dilute PTYG medium (as described above, but without agar), dilute PTYG medium with 0.5% PVP-360, and dilute PTYG medium with 2% PVP-360. Growth was then monitored turbidimetrically at 600 nm for 26 h at 23°C.

Quantitative and qualitative evaluation of cell recovery. The percentage of the viable microflora that was released into the films during flotation with PVP solutions was estimated with plate counts. Films were prepared with 0.5% PVP-360 as described above. The film and the residual pellet (left in the bottom of the Büchner funnel) were then collected separately and suspended in known volumes of sterile 0.1% $Na_4P_2O_7 \cdot 10H_2O$. These suspensions were blended for 1 min in a Waring blender. Cells were then enumerated by plating on dilute PTYG medium as described above. The number of viable cells in the film was compared with that in the pellet to determine the percent recovery. In addition, the colony types on film and pellet plates were analyzed and compared with those on plates from unaltered (i.e., not floated) sample material to determine whether the film contained a representative sampling of the microbial forms present in the original sample.

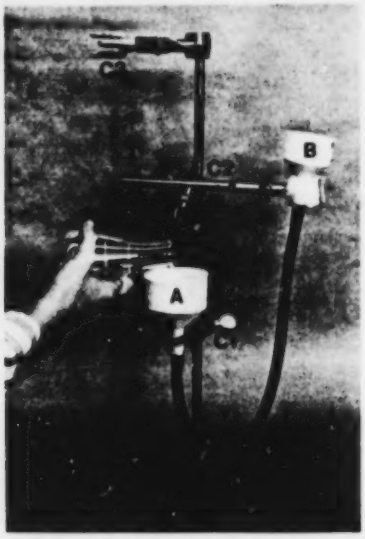

FIG. 1. Apparatus used for flotation procedure. Flotation fluid being poured into no. 3 Büchner funnel (A) before flotation procedure was done as described in the text. (Both funnels were normally covered with aluminum foil to prevent airborne contamination.) Also shown are no. 2 Büchner funnel (B) and burette clamps positioned at 12 (C1), 18 (C2), and 30 (C3) cm above the base of the ring stand.

FIG. 2. Transmission electron micrograph of metal-shadowed flotation film released from sample E9B/1. A dividing, rod-shaped bacterial cell (C) can be easily distinguished from the surrounding particulate debris (D). Bar = 0.5 μm.

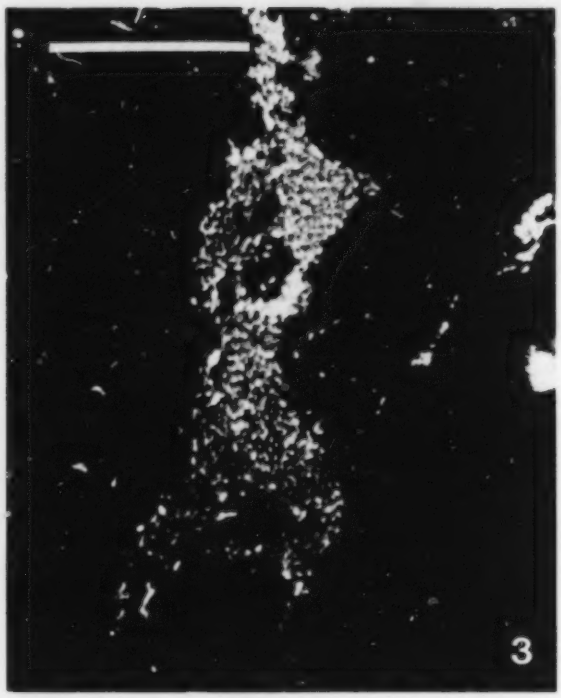

FIG. 3. Epifluorescence light micrograph of AO-stained flotation film released from sample 9U, depicting a large microcolony of rod-shaped cells. Bar = 20 μm.

RESULTS

Release of flotation films with PVP. A 0.5% solution of PVP-360 effectively released films from a variety of soils and sediments differing in saturation, physical composition, geographic source, and microbial population density (Table 1, samples E9B/1, E9B/2, E9B/3, 5G1B, 14J13, 16C4, 16D1, K1, and T1) and from a sample of limestone from a deep aquifer formation in southern Florida (provided by D. C. White, Florida State University). The film-releasing efficacy of the PVP solution was always equal to or better than that of distilled water alone, and it was considerably superior with most saturated samples.

PVP-produced films ranged in consistency from nearly transparent, thin pellicles to clearly visible layers of white or light brown material. These films all contained a transparent, highly cohesive, gellike substance but differed with respect to their content of opaque, particulate matter.

The efficiency of film release was not influenced by the molecular weight of the PVP used in the flotation fluid; 0.5% solutions of PVP-10, -40, and -360 all produced equal amounts of film. In contrast, the concentration of the PVP did affect film release. Film production declined at concentrations of PVP-360 below 0.5%, although 0.01% solutions still released noticeably more film than distilled water did. Increasing amounts of particulate matter were found in the films at concentrations above 2% (hindering subsequent microscopy), and the flotation fluids became inconveniently viscous. Concentrations from 0.5 to 2% provided acceptable results with most soil and sediment samples examined in this study. However, usable films were obtained from the limestone sample only at concentrations of 5% or higher, sug-

TABLE 2. Distinct morphological types in flotation films released from surface soils and subsurface sediments at two sampling sites

Morphological type	Occurrence (x) of morphological type in samples from[a]:						
	Albany, Ga.			Lula, Okla.			
	E9B/1 (0.25)	E9B/2 (0.65)	E9B/3 (1.20)	9U (—[b])	9S4 (0.22)	9S1 (1.32)	9S8 (7.57)
Cocci							
Single	x	x	x	x	x	x	x
In chains	x	x	x				
In sarcinal packets	x			x			
Large cells				x	x	x	x
Microcolonies	x			x	x		
Rods							
Small, straight, single	x	x		x	x	x	x
Small, straight, in chains	x			x		x	
In pairs	x			x	x		
Long, thin, curved, single		x		x			
Short, curved, single		x			x	x	
With tapered ends	x	x					
Large, with multiple inclusions	x			x			
Small, with single inclusions				x	x		
Coccoid				x	x	x	x
Coccoid, with inclusions				x	x	x	x
Microcolonies	x			x	x		
Actinomycetelike filaments				x	x		
Eucaryotic forms[c]:							
Oval-shaped yeasts, single	x			x	x		
Rod-shaped yeasts, single				x		x	
Microcolonies				x			

[a] Sample depth (meters) is given in parentheses after sample number; see Table 1 for further sample information.
[b] —, Surface soil.
[c] These forms contained densely stained nuclei.

gesting that optimum PVP concentrations should be established for each new type of sample examined.

Effect of PVP on microbial growth. PVP did not affect the growth of aquifer bacterial isolates pH2-B and 82-D. The growth rates and yields of cultures grown with 0.05 or 2% PVP-360 in the medium did not differ markedly from those of cultures grown without PVP.

Use of AO-stained films to survey microbial morphology.

TABLE 3. Microbial morphological diversity in soils and subsurface sediments, as assessed by light microscopy of AO-stained flotation films and by spread plating

Sample[a]	Depth (m)	No. of recognizably distinct cell morphologies in films	No. of recognizably distinct colony types on plates[b]
K1	—[c]	17	17
T1	—	19	17
E9B/1	0.25	11	5
E9B/2	0.65	6	5
E9B/3	1.20	2	2
9U	—	17	22
9S4	0.22	12	10
9S1	1.32	7	9
9S8	7.57	5	5

[a] Samples are grouped by sampling site; see Table 1 for further information.
[b] Containing dilute PTYG medium.
[c] —, Surface soil.

PVP-produced films were easily retrieved for light or electron microscopy because they adhered readily to glass slides, cover slips, coated grids, etc. Negatively stained or metal-shadowed films resembled the water-produced films described by Waid (11) and were equally suitable for TEM (Fig. 2). However, information on microbial morphology was derived from the films more rapidly when they were stained with AO and examined by epifluorescence light microscopy. Large areas of film could be scanned quickly at low magnifications (compared with those of TEM) because the microbial cells stood out clearly (Fig. 3) as bright green fluorescent objects against an orange-red background of particulate matter. Rapid scanning of large film areas was especially useful for generating morphological data on samples with low numbers of microbial cells.

Epifluorescence light microscopy of AO-stained films was a convenient method for rapidly assessing the morphological diversity of the resident microflora in soils and sediments. When it was used to compare surface soils to subsurface sediments at two different sampling sites, this approach effectively demonstrated that there was a marked decrease in diversity with increasing depth (Table 2, Fig. 4). Moreover, the data obtained by light microscopy of AO-stained films were comparable to the data provided by spread plating; the numbers of recognizably distinct cell types in the films paralleled the numbers of distinct colony types on the plates when both methods were applied to the same samples (Table 3).

In addition to fundamental cell morphology data, AO-stained films provided information on in situ spatial relationships among microbial cells. Because the microorganisms

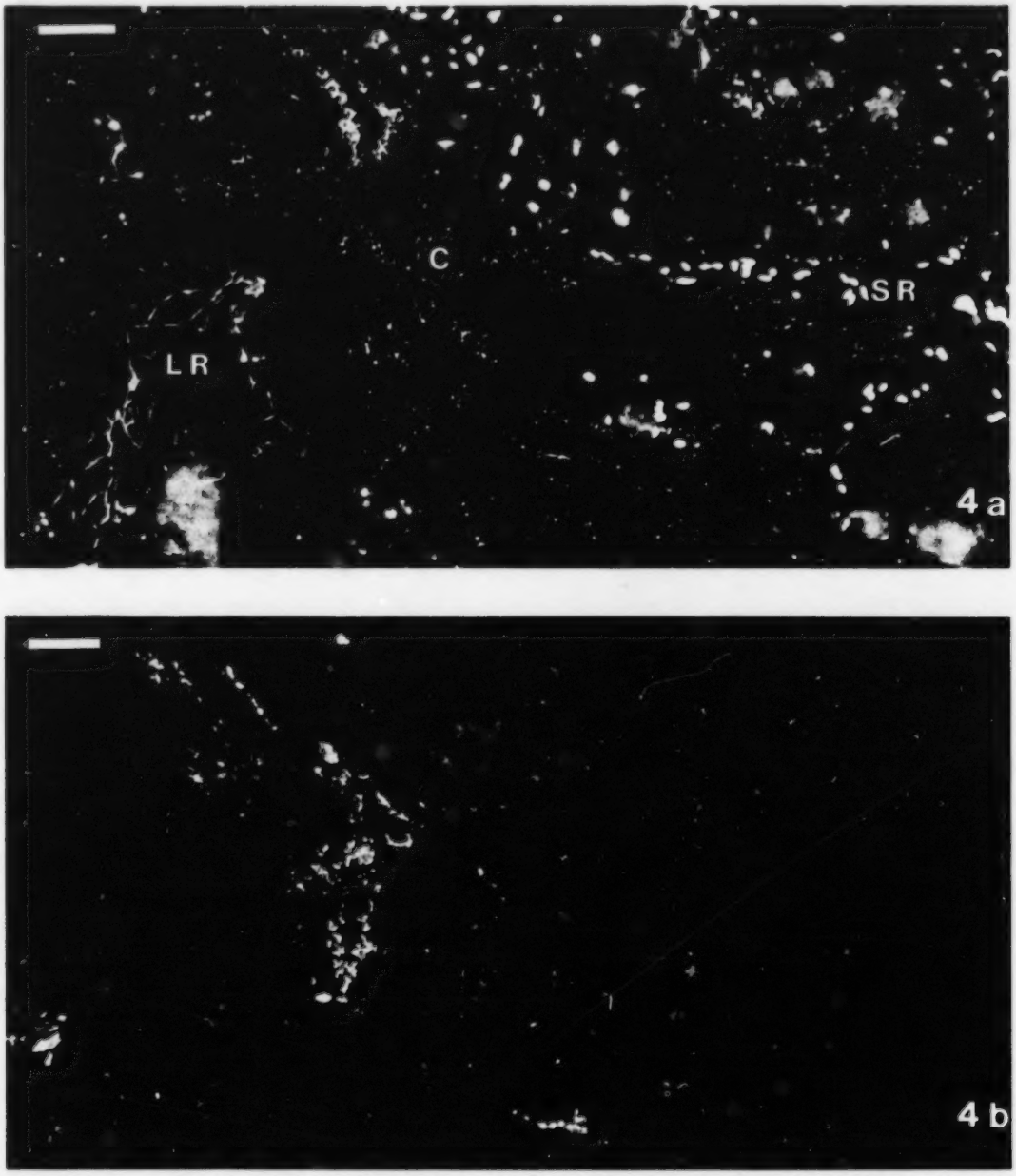

FIG. 4. Epifluorescence light micrographs of AO-stained flotation films released from samples taken from two different depths at Albany, Ga. Bars = 20 μm. (a) Field from the topsoil sample (E9B/1), showing diversity of morphological types. Note the presence of loose associations (see text) of coccoid cells (C), short rods (SR), and long rods (LR). (b) Field from the saturated subsurface sample (E9B/3). Note the lack of morphological diversity in comparison with that in the field from the topsoil sample.

were largely undisrupted by the flotation process, it was possible to detect microcolonies in which the constituent cells were in direct contact with each other (Fig. 3) and loose associations in which cells with identical morphological characteristics were not in direct contact but were grouped within a confined area of the film that contained no other cell types (Fig. 4). Light microscopy of AO-stained films from surface and subsurface samples from two sites revealed that the depth-related decline in morphological diversity described above (Tables 2 and 3) was accompanied by sharp decreases in the numbers of microcolonies and loose associations. The deepest sediments contained mostly single, unassociated microbial cells, whereas microcolonies and loose associations were common in the overlying surface soils.

Quantitative and qualitative aspects of the flotation process. The numbers of viable cells in PVP-produced films were compared with those in the pellets, yielding an estimation of

TABLE 4. Quantitative and qualitative evaluation of viable cell recovery during PVP flotation[a]

Sample[b]	No. of viable cells in[c]:		Recovery (%)	No. of distinct colony types in[d]:		
	Film	Pellet[e]		Film	Pellet	Soil[f]
T1	$1.0\ (0.1) \times 10^7$	$9.3\ (3.3) \times 10^7$	9.9	17	16	17
9U	$3.4\ (0.5) \times 10^6$	$9.7\ (1.0) \times 10^7$	3.4	15	15	15
9S8	$9.9\ (0.5) \times 10^6$	$8.8\ (2.7) \times 10^7$	10.1	9	10	ND[g]

[a] Determined on dilute PTYG medium.
[b] See Table 1 for sample descriptions.
[c] Total number of cells in the indicated fraction; average from triplicate spread plates with standard deviation given in parentheses.
[d] On plates at the statistically countable dilution or higher; except for the absent type on the T1 pellet and 9S8 film plates, the colony types on the film, pellet, and soil plates of each sample were identical.
[e] Material left in Büchner funnel after flotation (see Materials and Methods).
[f] Data from plating 1 g of unaltered (i.e., not floated) sample; viable counts for unaltered samples are given in Table 1.
[g] ND, No data.

the percentage of cells recovered during flotation. Plate counts showed that only a minority (3.4 to 10.1%) of the viable microbial cells were in the films (Table 4). However, analysis of colony types revealed that film plates contained essentially as many different kinds of colonies as did the equivalent pellet plates or plates of unaltered (i.e., not floated) sample material (Table 4). Moreover, the film, pellet, and unaltered soil plates from the same sample all contained the same types of colonies; there was no indication that certain colony types were lost during flotation. Similar results were obtained when surface soils that contained a wide variety of morphological types (samples 9U, K1, and T1) were examined by both AODC and flotation. All of the distinct cell types seen in the AODC preparations were also seen in the AO-stained flotation films.

DISCUSSION

The Waid flotation technique (8, 11, 12) quickly and effectively releases microbial cells from surface soils for subsequent observation by TEM but does not work well with saturated materials such as aquifer sediments. The present study showed that this problem can be solved by the substitution of solutions of PVP for water. Because it releases films effectively from both saturated and unsaturated samples, is inexpensive, and is nontoxic to microorganisms, PVP is recommended as a releasing agent for all types of flotation studies.

Waid observed flotation films only with TEM (11, 12). The capabilities of his method were expanded significantly in the present study by the use of light microscopy to observe PVP-produced films stained with AO. The comparatively low magnifications used in light microscopy allow observation of large numbers of cells quickly and cheaply; considerably more time and expense is required to view equal numbers of cells with TEM. Adding light microscopy to the Waid technique then makes it possible to determine rapidly the overall range of microbial morphological diversity in soils, even in subsurface samples with low cell numbers. TEM of PVP-produced films can then be used specifically to study cell features that are not resolved by the light microscope.

Flotation films viewed by light microscopy can also provide data on in situ (or at least relatively undisrupted) spatial relationships among cells because microcolonies and loose associations (see Results) remain intact during film production. Spatial information cannot be obtained with AODCs, plate counts, or release and concentration methods (see above) because these techniques involve extensive physical disruption of the sample, yet such data can be valuable as indirect indicators of in situ microbial physiological states.

Only a small fraction (3.4 to 10.1%) of the microflora was recovered in flotation films by the method developed in this study. If the method is to be useful as a tool for studying morphological diversity and spatial relationships, it is important that this small sampling of cells be representative of the total population. Analyses of colony types demonstrated that this was clearly the case for platable microorganisms. A representative sampling of the nonplatable flora was probably obtained as well because all of the recognizably distinct cell types seen by AODC, a method that does not have separation steps in which a portion of the microflora could be lost, were also detected in AO-stained flotation films. This would not have been the case if the flotation process were selective for certain cell types.

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